Nano Vision, Vol.1 (1), 1-13 (2011)

Macrophage and Osteoblast Response to Micro and Nano Hydroxyapatite – A Review

S. BABY GAYATHRI and KAMARAJ P.*

*Department of Chemistry, Faculty of Engineering and Technology,
SRM University, Kattankulathur, Tamil Nadu- 603203, India.
email: kamaraj97@yahoo.co.in

ABSTRACT

Bone is a connective tissue formed by the precipitation of hydroxyapatite crystals on organic matrix. One of the most common illnesses associated with the bone is bone fracture. The use of implant at the affected bone region is found to be associated with several difficulties especially due to the activity of macrophage and osteoblast at the implanted site. Osteoblasts are specialized fibroblast-like cells associated with the formation of bone and macrophages, members of host immune system. Orthopedic surgery is known to trigger a wound healing response at the implanted site by activation of numerous immune cells long before osteoblasts arrive. Unique higher surface area, surface roughnesses, altered electron distribution and energetics of nano-hydroxyapatite have altered the activity of macrophage and osteoblast when coated over a substrate. An overview of macrophage and osteoblast response to micro and nano-hydroxyapatite that is involved in achieving maximal bone response is discussed.

Keywords: Hydroxyapatite, nano-hydroxyapatite, osteoblast, macrophage.

INTRODUCTION

Bone is an extremely dynamic tissue, formed by the precipitation of hydroxyapatite (HA) crystals on organic protein matrix. HA crystals are formed when the body cells tend to store calcium and phosphorus in the extracellular matrix. The precipitation of HA crystals on collagen matrix forms bone tissue[1]. Besides its significance in biology, HA is also a good candidate for applications as catalyst[2] and gas sensors[3]. One of the notable bone illnesses is bone fracture which can be healed upon tended and supported implant. On failure, bone fracture may lead to

misgrowth of bone[4]. In the past several years, many cases have been reported on bone implants and the associated difficulties especially due to macrophage and osteoblast responses over the implant.

Osteoblasts are specialized fibroblast-like cells of primitive mesenchymal origin called osteoprogenitor cell that originate from pluripotent mesenchymal stem cells of the bone marrow. The evidence of mesenchymal stem cells as precursors for osteoblasts is based on the capacity of bone to regenerate itself both in vivo and in vitro by using cell populations[5]. It has been shown that the bone marrow stroma have the capacity to differentiate into osteoblasts, chondroblasts, fibroblasts, adipocytes and myoblasts[6]. Macrophages are specialized phagocytes that play an important role in clearance of effete host cells and molecules[7], as well as in defense against foreign invasion, including infection, allogenic or xenogenic transplantation and implantation. Orthopedic surgery is known to cause trauma to the local tissue triggering a wound healing response activating numerous inflammatory cells at the implant site long before osteoblasts arrive. One type of inflammatory cell (macrophages) migrates to the bone implant interface and becomes activated soon following implantation[8]. Macrophages are the first line of defense against bacteria, viruses and foreign implanted materials. Overactivation of macrophages can lead to the production of pro-inflammatory cytokines (IL-1B, IL-6), Chemokines (CCL22), matrix enzymes (elastase) and other substances (PGE2), which not only can cause osteolysis but can also stimulate the proliferation of fibroblasts[9,10]. This would decrease the ability of osteoblasts to attach proliferate and form bone on orthopedic implants.

Macrophages are found to actively respond to almost all implants in vivo, including metal[11], ceramics and cements[12], polymers[13,14], and protein material such as collagen[15,16] etc. As macrophages are members of the host immune system, their responses to biomaterials have attracted wide concern. HA being biocompatible, when coated over the implant can down-regulate the macrophage activities and migration so that wound healing can occur quickly and bone growth and mineral deposition by osteoblasts are prompted shortly there after. Controlling surface feature size and corresponding surface roughness on implants may clearly alter immune cell responses, which would be an extremely important consideration for the use of nanostructured materials as improved biomaterials. In addition, incorporation of nanoparticles over the implant has been found to decrease the macrophage proliferation[17] and increase osteoblast adhesion[18] at the implanted site. The objective of this review is to discuss on the use of micro and nano- HA coated implants and to bring the macrophage and osteoblast reaction at the implanted site. For an implant to be biocompatible, the implant-bone interface should mimic with the interfaces naturally occurring between bones and tendons and ligament.

MACROPHAGE AND OSTEOBLAST RESPONSE TO HA

Many studies have been performed regarding the macrophage proliferation and osteoblast adhesion on different materials that were used as surgical implants[19].

Macrophage initiates the entire process of phagocytosis ultimately decreasing the ability of the osteoblast cells to get adhered at the implant surface. The adverse effect of synthetic compounds on growth disturbance, migration, infection, rigidity, effects on cellular level[20] is reduced by the use of HA in the implant.

MACROPHAGE AND WOUND HEALING PROCESS

The overall inflammatory kinetics and their mediators in activating the cells at the implanted site are not fully understood. The activation of macrophage is initially through an innate immune response. This involves a combination of tumor-necrosis factor alpha (TNF-α) and interferon-γ (IFNγ) to promote a bactericidal phenotype (e.g., expression IL-1, IL-6 and IL-23)[21]. Later, these cells respond to both innate and adaptive immune response[22] involving response to basophil and mast cell release of IL-4 eliciting a differentiation of macrophage into a wound healing pathway[23]. Adaptive immune response occurs though TH2 helper cell and IL-10 expression leading to expression from an intermediate regulatory macrophage of IL4 and IL-13. This cytokine regulated cellular recruitment, migration, proliferation and formation of an extracellular matrix on the implant surface can be influenced by this early population of macrophages[24]. These cells express growth factors such as fibroblast growth factor (FGF-1, FGF-2, FGF-4), Transforming growth factors, epithelial growth factor as well as bone morphogenetic proteins (BMPs)[25]. The end result of this complex cascade is promotion of a wound healing process that includes angiogenesis[26]. The development of an elaborate vascular network is an important part of the implant wounding healing process and may be elicited by the initial ischemia in the immediate wound site followed by the macrophage mediated release of bFGF, TNF-α and vascular endothelial growth factor (VEGF)[27].

OSTEOINTEGRATION AND OSTEOBLAST DIFFERENTIATION

The role of macrophage in implant topography has primarily focused on implanted material and potential for inflammation in the vascular based devices. One group of scientists believes that the induction of macrophage apoptosis can eliminate the inflammation caused due to these reactions[28]. At the other hand, human osteoblast cells were able to differentiate and proliferate in vitro over HA composites[29, 30]. Phagocytosis of wear debris by osteoblasts affects differentiation and local factor production in a manner dependent on particle composition at the implanted site[31].

The subsequent formation of a mineralized matrix during osteogenesis and bone remodeling or during osteointegration of HA composites involves the recruitment of multipotent mesenchymal stem cells and the progressive differentiation of these cells into osteoblasts[32]. Osteoblast differentiation and skeletal formation during embryonic development is mediated by an essential transcription factor protein called core binding-factor-alpha (Cbfa1)[34]. Cbfa1 belongs to the Runt family of transcription factors[32], and regulates osteoblast differe-ntiation and expression of bone extracellular matrix protein genes that encode for bone sialoprotein [BSP], Osteocalcin and Type I

Collagen[35,36]. Cbfa1 plays an essential role in osteogenesis, osteoblast matrix formation, chondrocyte differentiation, and bone resorption by osteoclasts[37], and could therefore be a downstream target of cellular events such as extracellular matrix adhesion-mediated signaling, changes in cell shape and responses to local paracrine environments. A second transcription factor, osterix, has been described and has been suggested to play a key role downstream of Cbfa1 in which its expression is necessary for the ongoing differentiation within in the osteogenic pathway[38].

CELLULAR RESPONSE ON HA

The non-toxic and natural degradation of HA make it an ideal candidate for in vivo application[39]. However the modes of synthesis have been found to be associated with the degradation in vitro and in vivo. The HA which was deposited by ion-beam technique was less degradable when compared to the HA coated via plasma spray deposition technique in vitro. It should be noted that the rapid degradability of the implanted material will lead to shortening the longitivity of the implant[40]. Hence HA coated via ion-beam technique is more stable. The in vivo study does not show any significant difference in degradability but the adhesiveness of the osteoblast was reported to be higher in Ion beam deposited HA coating[41]. In a comparative study performed by Nausa et al performed on the tissue response to two synthetic HA which differ in their crystallinity (20% and 70%), there was no significant difference in the quality and quantity of the inflammatory cells at the implanted site in vitro[42]. HA which are highly crystalline are chemically stable and interfere with osteoblast cell proliferation by decreasing in vitro cell adhesion[43, 44].

The physiochemical properties of HA do not affect the cellular response in vitro[42]. In vivo study on the tissue response for the various crystalline HA has to be conducted to agree or disagree on the stated inference. Added to this, the association of cellular response to HA synthesized and deposited via various methods along with its crystalline nature have to be well investigated.

MACROPHAGE AND OSTEOBLAST RESPONSE TO NANO-HA

HA are now being widely used in orthopedic implants[45,46,47] due to their biological characteristics such as non-immunogenicity, non-inflammatory behavior, greater biocompatibility and high osteoconductivity[48]. Atomic force microscopy of trabecular bone showed plate or ball shaped minerals (30 to 120 nm diameters) which were densely packed into woven collagen fibrils[49]. Ultrastructural examination of deproteinated bone reveals that individual 25–50 nm HA crystals are the essence of bone in terms of mechanical properties and bioresorbability, and play an important role in biomineral formation[50]. Thus, nanosized HA particles have aroused intensive interest, and great efforts have been made in the last decade to study their synthesis, structure and properties[51,52]. Nano-HA has been proven to exhibit a better biocompatibility, good bioactivity and flexible structure[53,54,55] than micro-HA.

The surface roughness of the implant has a direct role in the biological response at the implanted site[18,56-60].

Nanoparticles when coated over the implant increase the surface roughness which provides greater osteoblast adhesion[18,56]. The molecular interactions at the surface can be targeted to create specific cell level response[57,58,59]. An increase in osteoblast adhesion and differentiation was demonstrated on HA nanoparticles and there was a significant reduction in its biological property with the increase in grain size of the particles[18,53-56,60,61]. Zhongli Shi *et al.* showed the variation in the osteoblast adhesion over 20nm, 80nm and micro-HA particle. 20nm HA (Figure 1) shows a greater cell adhesion than 80 nmHA and micro-HA[56]. Similar effects were noticed on few nanophase ceramics[22,23,24].

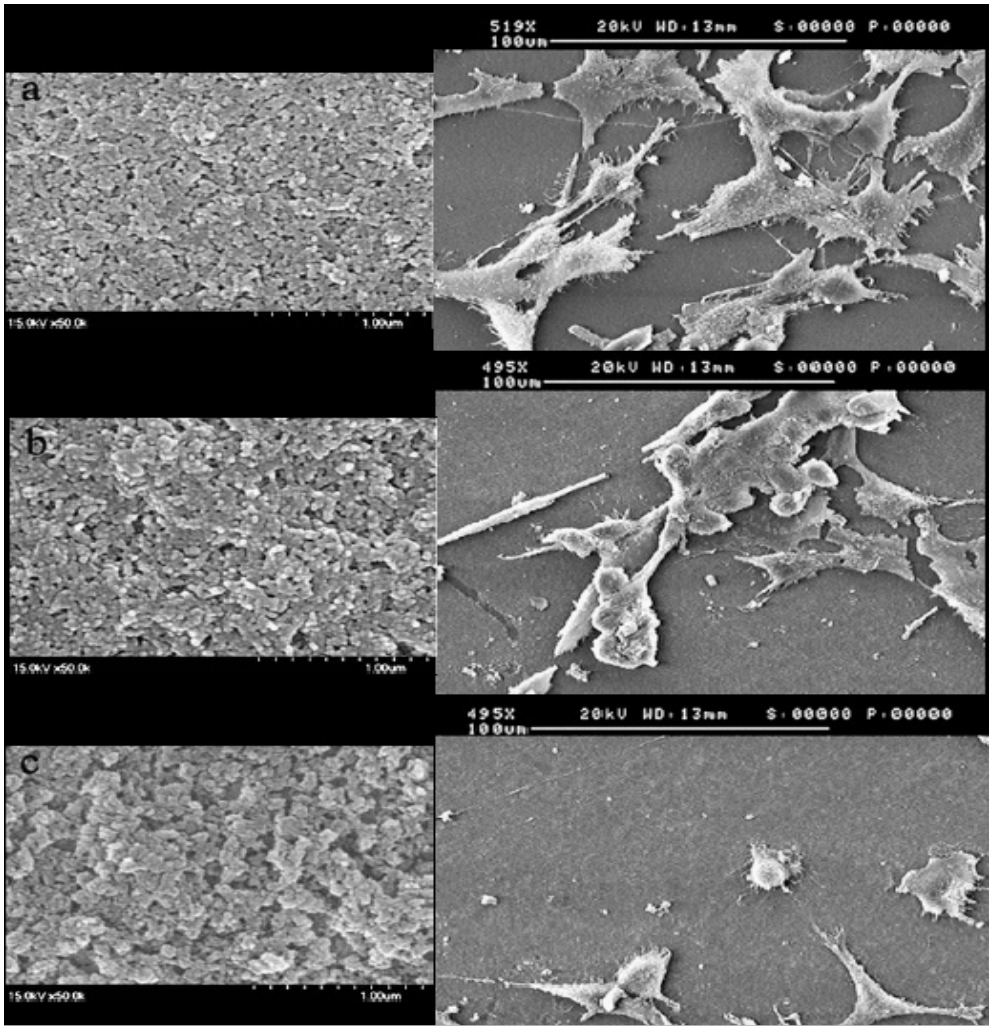

Figure 1: FESEM images of (a) 20nm, (b) 80nm and (c) micro-HA.

The corresponding images show the FESEM images of cellular morphology over HA particles. Cell proliferation was greater over 20nm sized HA particles[56].

The increase in biological activity over the effect of nanoparticles may be due to the orientation of protein to the nanoparticles and specifically the mode of orientation of adhesion proteins such as vitronectin to the grain boundaries which in turn alters osteoblast adhesion and shape; both critical to formation of bone[62,65,66]. Nano-HA can possibly stimulate osteoblastic proliferation compared with m-HA[67]. This may be ascribed to the enhanced interfacial adhesion of HA nanoparticles to cells, as well as the improved penetration abilities of smaller HAs. Due to the smaller size of the HA particles, they can penetrate into the cells more easily by endocytosis, clathrin-coated vesicles (pits), caveolae or their independent reporters[68] and stimulate growth factors. The origin of the effects of nanoparticles may be derived from the fact that the internalized HA nanoparticles were partially dissolved during lysosomal digestion and the obtained solutes such as Ca^{2+} ions diffused into the cytoplasm[69]. The proper Ca^{2+} concentrations favored osteoblast proliferation and differentiation[70].

There is a limited knowledge on the effect of crystallinity and mode of synthesis of nanoparticles on biological behaviors. The knowledge on the effect of crystallinity is more on m-HA than nano-HA[71,72]. The improvement in cell adhesive and differentiation properties by the nano-HA have been explored where lower crystallinity was found to enhance cell adhesion[73]. In addition nano-HA which were spherical in shape showed an enhanced adhesive property than rod shaped HA nanoparticles[74]. The explanation for the favorable effect of sphere-like nano-HA on osteoblasts might be the well-organized surface which seems beneficial for filopodia protrusion.

In the comparative study by Zhongli Shi *et al.*[56], on 20nm and 80nm HA particles, loss of contact with neighboring cells, contraction of the cells, swollen mitochondria, deformed nuclei and condensed chromatin was observed over 80nm HA particles (Figure 1). This proves the cytotoxic effect of larger size nanoparticles and the use of smaller size nanoparticles of HA. Controlling the size of HA nanoparticles is the major issue that has to be considered to eliminate this cytotoxic effect. In addition, nano-HA were found to be cytotoxic to macrophage and hence there is a decrease in macrophage proliferation over nano-HA[15].Motskin *et al.* demonstrated that HA particle toxicity is due to the increased cytosplasmic calcium load leading to cell death. Few nano-HA was found to enter the nuclei through nuclear pores.

CONCLUDING REMARKS

Macrophage and osteoblast response to micro and nano-HA is quite controversial. Macrophage is a member of immune system and its activities are closely related to the immune response, inflammation and foreign body response. On the other hand, osteoblasts are mononucleate cells, responsible for bone formation. The type of response by macrophage and osteoblast over HA depends on the size, crystallanity, surface roughness of the particles, etc. Osteoblast adhestion is enhanced when the macrophage proliferation is less. However, no study has been carried till now to find the

response of osteoblasts and macrophage on nano-HA simultaneously in vitro as well as in vivo. The in vitro and in vivo studies of osteoblast and macrophage response to micro-HA have proved it to be biocompatible due to its physiochemical properties. On incorporation of nano-HA the osteoblast adhesion was found to be significantly greater due to increased surface area and roughness. It should be noted that the crystallinity of the compound is inversely proportional to the biocompatibility and mechanical strength. HA however biocompatible has a less tensile strength. Hence HA is either coated over a substrate or HA composite is formed to enhance the mechanical properties.

It can be concluded that nano-HA may be a potential agent to enhance the osteoblast adhesion and decrease the macrophage activity. Hematopoiesis or blood cell formation occurs at the bone marrow. Hence it is necessary to find the kinetics of blood cells formed by the incorporation of nano-HA in the implant. However, the overall study on the response of osteoblast and macrophage over nano-HA, particle-osteoblast interaction and particle-macrophage interaction could pave more way for the future research and better application of nano-HA in bone implants. The study on particle-cell interaction could explain more about the biological stability derived from the design and surface of HA particles along with its association at the particle cell interface in aiding the attachment of bone forming cells with minimal inflammatory infiltrate.

REFERENCES

1. E. Dogan, and C. Posaci, "Monitoring hormone replacement therapy by biochemical markers of bone metabolism in menopausal women," *Postgrad Med J*, vol. 78, no. 926, pp. 727-731, (2002).
2. S. B. Gayathri and P. Kamaraj, "Catalytic Activity of Nano-Hydroxyapatite on the Production of Gasoline", *Proceedings of International Conference on Advanced Nanomaterials and Nanotechnology*, D-206, (2009).
3. S. B. Gayathri and P. Kamaraj,. "Resistance response models for Nano-Calcium Deficient Gas Sensors", *Proceedings of 98th Indian Science Congress- Chemical Sciences Section*, pp. 155, (2011).
4. S. Bandyopadhyay-Ghosh, "Bone as a Collagen-hydroxyapatite Composite and its Repair," *Trends Biomater Artif Organs*, Vol. 22, No. 2, pp. 116-124, (2008).
5. G.S. Stein, and J.B. Lian, "Molecular mechanisms mediating proliferation/ differentiation interrelationships during progressive development of the osteoblast phenotype," *Endocrine Review*, Vol. 14, No. 4, pp. 424-442, (1993).
6. A.J. Friedenstein, "Precursor cells of mechanocytes," *Int Rev Cytol*, Vol. 47, No.1, pp. 327-359, (1976).
5. S. Gordon, "Pattern recognition receptors: doubling up for the innate immune response," *Cell*, Vol. 111, No. 7, pp. 927–30, (2002).
6. J. E. Park, and A. Barbul, "Understanding the role of immune regulation in wound healing," *Am J Surg*, Vol. 187, no. 5, pp. 11S–6S, (2004).
7. P. E. Purdue, P. Koulouvaris, H.G. Potter, B.J. Nestor, and T.A. Sculco, "The cellular and molecular biology of

periprosthetic osteolysis," *Clin Orthop Rel Res*, Vol. 454, No. 1, pp. 251-261, (2007).

8. E. Ngham, and J. Fisher, "The role of macrophages in osteolysis of total joint replacement," *Biomaterials*, Vol. 26, No. 11, pp. 1217-1286, (2005).
9. J. Takebe, C. M. Champagne, S. Offenbacher, K. Ishibashi and L. F. Cooper, "Titanium surface topography alters cell shape and modulates bone morphogenetic protein 2 expression in the J774A.1 macrophage cell line," *J Biomed Mater Res*, Vol. 64A, No. 2, pp. 207-216, (2003).
10. J. Lu, M. Descamps, J. Dejou, G. Koubi, P. Hardouin, J. Lemaitre and J. P. Proust, "The biodegradation mechanism of calcium phosphate biomaterials in bone," *J Biomed Mater Res.,* Vol. 63, No. 4, pp. 408-412, (2002).
11. R. S. Labow, S. Danne, L.A. Matheson and J. P. Santerre, "Polycarbonate-urethane hard segment type influences esterase substrate specificity for human-macrophage-mediated biodegradation," *J Biomater Sci Polym Ed*, Vol. 16, No. 9, pp. 1167-1177, (2005).
12. E. Solheim, B. Sudmann, G. Bang and E. Sudmann, "Biocompatibility and effect on osteogenesis of poly(ortho ester) compared to poly(DL-lactic acid)," *J Biomed Mater Res*, Vol. 49, No. 2, pp. 257-263, (2000).
13. I. M. Khouw, P. B. van Wachem, L. F. de Leij and M. J. van Luyn, "Inhibition of the tissue reaction to a biodegradable biomaterial by monoclonal antibodies to IFN-gamma," *J Biomed Mater Res*, Vol. 41, No.2, pp. 202-210, (1998).
14. Z. D. Xia, T. B. Zhu, J. Y. Du, Q. X. Zheng, L. Wang, S. P. Li, C. Y. Chang and S. Y. Fang, "Macrophages in degradation of collagen/hydroxylapatite (CHA), beta-tricalcium phosphate ceramics (TCP) artificial bone graft. An in vivo study," *Chin Med J (Engl)*, Vol. 107, No. 11, pp. 845-849, (1994).
15. D. Khang, P. Liu-Snyder, R. Pareta, J. Lu and T. J. Webster, "Reduced responses of macrophages on nanometer surface features of altered alumina crystalline phases," *Acta Biomater*, Vol. 5, No. 5, pp. 1452-1432, (2009).
16. M. Nelson, G. Balasundaram, and T. J Webster, "Increased osteoblast adhesion on nanoparticulate crystalline hydroxyapatite functionalized with KRSR," *Int J Nanomedicine*, Vol. 1, No. 3, pp. 339-349, (2006).
17. G. M. Kontakis, J. E. Pagalos, T. I. Tosounidis, J. M. Melissas, and P. Katonis, "Bioabsorbable materials in orthopaedics," *Acta Orthop Belg*, Vol 73, No. 2, pp. 159-169, (2007).
18. E. Ingham, and J. Fisher, "Biological reactions to wear debris in total joint replacement'" *Proc Inst Mech Eng H*, Vol. 214, No. 1, pp. 21-37, (2000).
19. D. M. Mosser, and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nat Rev Immunol*, Vol. 8, No. 12, pp. 958-969, (2008).
20. P. Loke, I. Gallagher, M. G. Nair, X. Zang, F. Brombacher, M. Mohrs, J. P. Allison, and J. E. Allen, "Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection," *J*

Immunol, Vol. 179, No. 6, pp. 3926-3936, (2007).

21. E. Brandt, G. Woerly, A. B. Younes, S. Loiseau, and M. Capron, "IL-4 production by human polymorph-onuclear neutrophils," *J Leukoc Biol*, Vol. 68, No. 1, pp. 125-130, (2000).
22. M. Crowther, N. J. Brown, E. T. Bishop, and C. E. Lewis, "Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors," *J Leukoc Biol*, Vol. 70, No. 4, pp. 478-490, (2001).
23. T. A. Linkhart, S. Mohan, and D. J. Baylink, "Growth factors for bone growth and repair: IGF, TGF beta and BMP," *Bone*, Vol. 19, No. 1. Pp. 1S-12S, (1996).
24. J.S. Lewis, J. A. Lee, J. C. Underwood, A. L. Harris, and C. E. Lewis, "Macrophage responses to hypoxia: Relevance to disease mechanisms," *J Leukoc Biol*, Vol. 66, No. 6, pp. 889-900, (1999).
25. T. Okazaki, S. Ebihara, H. Takahashi, M. Asada, A. Kanda, and H. Sasaki, "Macrophage colonystimulating factor induces vascular endothelial growth factor production in skeletal muscle and promotes tumor angiogenesis" *J Immunol*, Vol. 174, No. 12, pp. 7531-7538, (2005).
26. A. Petit, I. Catelas, J. Antoniou, D. J. Zukor, and O. L. Huk, "Differential apoptotic response of J774 macrophages to alumina and ultrahigh- molecular-weight polyethylene particles," *J Orthop Res*, Vol. 20, No.1, pp. 9-15, (2002).
27. F. B. Bagambisa, and U. Joos, "Preliminary studies on the phenomenological behaviour of osteoblasts cultured on hydroxyapatite ceramics," *Biomater*, Vol. 11, No. 1, pp. 50-56, (1990).
28. C. M. Holden, and G. W. Bernard, "Ultrastructural in vitro characterization of a porous hydroxyapatite/bone cell interface," *J Oral Implantol*, Vol. 16, No. 2, pp. 86-95, (1990).
29. C. H. Lohmann, Z. Schwartz, G. Koster, U. Jahn, G. H. Buchhorn, M. J. MacDougall, D. Casasola, Y. Liu, V. L. Sylvia, D. D. Dean, and B. D. Boyan, "Phagocytosis of wear debris by osteoblasts affects differentiation and local factor production in a manner dependent on particle composition," *Biomater*, Vol. 21, No. 6, pp. 551-561, (2000).
30. J. E. Aubin, F. Liu, L. Malaval, and A. K. Gupta, "Osteoblast and chondroblast differentiation," *Bone*, Vol, 17, No. 2, pp. 77S-83S, (1995).
31. P. Ducy, R. Zhang, V. Geoffroy, A. L. Ridall, and G. Karsenty, "Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation," *Cell*, Vol. 89, No. 5, pp. 747-754, (1997).
32. G. Xiao, D. Wang, M. D. Benson, G. Karsenty, and R. T. Franceschi, "Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor," *J Biol Chem*, Vol. 273, No. 43, pp. 32988-32994, (1998).
33. H. Harada, S. Tagashira, M. Fujiwara, S. Ogawa, T. Katsumata, A. Yamaguchi, T. Komori, and M. Nakatsuka, "Cbfa1 isoforms exert functional differences in osteoblast differentiation," *J. Biol. Chem.* Vol. 274, no. 11, pp. 6972-6978, 1999.

34. P. Ducy, M. Starbuck, M. Priemel, J. Shen, G. Pinero, V. Geoffroy, M. Amling, and G. A. Karsenty, "A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development," *Genes dev*, Vol. 13, No. 8, pp. 1025-1036, (1999).
35. K. Hoshi, T. Komori, and H. Ozawa, "Morphological characterization of skeletal cells in Cbfa1 deficient mice," *Bone*, Vol. 25, No. 6, pp. 639-651, (1999).
36. K. Nakashima, X. Zhou, G. Kunkel, Z. P. Zhang, J. M. Deng, R. R. Behringer, and B. de Crombrugghe, "The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation," *Cell*, Vol. 108, No. 1, pp. 17-29, (2002).
37. J. T. Edwards, J. B. Brunski, and H. W. Higuchi, "Mechanical and morphologic investigation of the tensile strength of a bone-hydroxyapatite interface," *J Biomed Mater Res*, Vol. 36, No. 4, pp. 454-468, (1997).
38. J. D. de Bruijn, "Calcium phosphate biomaterials: bone-bonding and biodegradation properties," *Ph.D. Thesis, University of Leiden, Leiden, Netherlands*, 1993.
39. Z. S. Luo, F. Z. Cui, Q. L. Feng, H. D. Li, X. D. Zhu, and M. Spector, "In vitro and in vivo evaluation of degradability of hydroxyapatite coatings synthesized by ion beam-assisted deposition," *Surf Coating Tech*, Vol. 131, No. 1-3, pp. 192-195, (2000).
40. N. M. de Freitas Costa, B. R. Melo, R. T. Brito, G. V. de Oliveira Fernandes, V. G. Bernardo, E. C. Fonseca, M. B. Conz, G. A. Soares, and J. M. Granjaro, "Quality and Intensity of the Tissue Response to Two Synthetic Granular Hydroxyapatite Implanted in Critical Defects of Rat Calvaria," *Mat Res*, Vol. 12, No. 2, pp. 245-251, (2009).
41. Y. Yang, D. Dennison, and J. L. Ong, "Protein adsorption and osteoblast precursor cell attachment to hydroxyapatite of different crystallinities," Int *J Oral Maxillofac Implants*, Vol. 20, No. 2, pp. 187-192, (2005).
42. L. Chou, B. Marek B, and W. R. Wagner, "Effects of hydroxyapatite coating crystallinity on biosolubility, cell attachment efficiency and proliferation in vitro," *Biomater*, Vol. 20, No. 10, pp. 977-985, (1999).
43. S. Wojciech, and Y. Masahiro, "Processing and properties of hydroxyapatite- based biomaterials for use as hard tissue replacement implants" *J Mater Res*, vol. 13, no. 1, pp. 94-117, 1998.
44. S. F. Hulbert, J. C. Bokros, L. L. Hench, J. Wilson, and G. Heimke, "Ceramics in clinical applications, past, present and future, in High Tech Ceramics," P. Vincenzini, ed. *Elsevier*, Amsterdam, pp. 3-27, (1987).
45. H. Oonishi, "Orthopaedic applications of hydroxyapatite," *Biomater*, Vol. 12, No. 2, pp. 171-178, (1991).
46. J. C. Hornez, F. Chai, F. Monchau, N. Blanchemain, M. Descamps, and H. F. Hildebrand, "Biological and physico-chemical assessment of hydroxyapatite (HA) with different porosity," *Biomol Eng*, Vol. 24, No. 5, pp. 505-509, (2007).
47. T. Hassenkam, G. E. Fantner, J. A. Cutroni, J. C. Weaver, D. E. Morse, P.

K. Hansma, "High-resolution AFM imaging of intact and fractured trabecular bone," *Bone*, Vol. 35, No. 1. Pp. 4-10, (2004).

48. M. J. Olszta, X. Cheng, S. S. Jee, R. Kumar, Y. Y. Kim, M. J. Kaufman, E. P. Douglas and L. B. Gower, "Bone structure and formation: a new perspective," *Mater Sci Eng*, Vol. 58. No. 3-5, pp. 77–116, (2007).
49. M. J. Phillips, J. A. Darr, Z. B. Luklinska, and I. Rehman, "Synthesis and characterization of nano-biomaterials with potential osteological applications," *J Mater Sci Mater Med*, Vol. 14, No. 10, pp. 875-882, (2003).
50. S. A. Catledge, M. D. Fries, Y. K. Vohra, W. R. Lacefield, J. E. Lemons, S. Woodard, and R. Venugopalan, "Nanostructured ceramics for biomedical implants," *J Nanosci Nanotechnol*, Vol. 2, No. 3-4, pp. 293-312, (2002).
51. J. Huang, Y. W. Lin, X. W. Fu, S. M. Best, R. A. Brooks, N. Rushton, and W. Bonfield, "Development of nano-sized hydroxyapatite reinforced composites for tissue engineering scaffolds," *J Mater Sci Mater Med*, vol. 18, no. 11, pp. 2151-2157, (2007).
52. F. Chen, W. M. Lam, C. J. Lin, G. X. Qiu, Z. H. Wu, K. D. Luk, and W. W. Lu, "Biocompatibility of electroph-oretical deposition of nanostructured hydroxyapatite coating on roughen titanium surface: in vitro evaluation using mesenchymal stem cells," *J Biomed Mater Res B Appl Biomater*, Vol. 82, No. 1, pp. 183-191, (2007).
53. K. U. Lewandrowski, S. P. Bondre, D. L. Wise, and D. J. Trantolo, "Enhanced bioactivity of a poly (propylene fumarate) bone graft substitute by augmentation with nano-hydroxy-apatite," *Biomed Mater Eng*, vol. 13, no. 2, pp. 115-124, (2003).
54. Z. Shi, X. Huang, Y. Cai, R. Tang, and D. Yang, "Size effect of hydroxyapatite nanoparticles on proliferation and apoptosis of osteoblast-like cells," *Acta Biomater*, Vol. 5, No. 1, pp. 338-345, (2009).
55. L. Meirelles, L. Melin, T. Peltola, P. Kjellin, I. Kangasniemi, F. Currie, M. Andersson, T. Albrektsson, and A. Wennerberg, "Effect of hydroxyapatite and titania nanostructures on early in vivo bone response," *Clin Implant Dent Relat Res*, Vol. 10, No. 4, pp. 245-254, (2008).
56. G. Mendonca, D. B. Mendonca, L. G. Simoes, A. L. Araujo, E. R. Leite, W. R. Duarte, L. F. Cooper, and F. J. Aragao, "Nanostructured alumina-coated implant surface: Effect on osteoblast-related gene expression and bone-to-implant contact in vivo," *Int J Oral Maxillofac Implants*, vol. 24, no. 2, pp. 205-215, (2009).
57. G. Mendonca, D. B. Mendonca, L. G. Simoes, A. L. Araujo, E. R. Leite, W. R. Duarte, F. J. Aragao, and L. F. Cooper, "The effects of implant surface nanoscale features on osteoblast-specific gene expression," *Biomater*, Vol. 30, No. 25, pp. 4053-4062, (2009).
58. L. Meirelles, T. Albrektsson, P. Kjellin, A. Arvidsson, V. Franke-Stenport, M. Andersson, F. Currie, and A. Wennerberg, "Bone reaction to nano hydroxyapatite modified titanium implants placed in a gap-healing

model," *J Biomed Mater Res A,* vol. 87, no. 3, pp. 624-631, 2008.

59. M. Sato, A. Aslani, M. A. Sambito, N. M. Kalkhoran, E. B. Slamovich, and T. J. Webster, "Nanocrystalline hydroxyl-apatite/ titania coatings on titanium improves osteoblast adhesion", *J Biomed Mater Res A,* Vol. 84, No. 1, pp. 265-272, (2007).
60. T. J. Webster, and E. S. Ahn, "Nanostructured biomaterials for tissue engineering bone," *Adv Biochem Eng Biotechnol,* Vol. 103, No. 1, pp. 275-308, (2007).
61. G. Mendonca, D. B. Mendonca, F. J. Aragao, and L. F. Cooper, "Advancing dental implant surface technology: From micron-to nanotopography," *Biomater,* Vol. 29, No. 28, pp. 3822-3835, (2008).
62. T. J. Webster, R. W. Siegel, and R. Bizios, "Design and evaluation of nanophase alumina for orthopaedic/ dental applications," *Nanostruct Mater,* Vol. 12, No. 5, pp. 983-986, (1999).
63. G. Balasundaram, C. Yao, and T. J. Webster, "TiO2 nanotubes function-alized with regions of bone morph-ogenetic protein-2 increases osteoblast adhesion," *J Biomed Mater Res A,* Vol. 84, No. 2, pp. 447-453, (2008).
64. G. Balasundaram, and T. J. Webster, "An overview of nano-polymers for orthopedic applications" *Macromol Biosci,* Vol. 7, No.5, pp. 635-662, (2007).
65. X. Guo, J. E. Gough, P. Xiao, J. Liu, and Z. Shen, "Fabrication of nanostructured hydroxyapatite and analysis of human osteoblastic cellular response," *J Biomed Mater Res A,* Vol. 82, No. 4, pp. 1022-1032, 2007.
66. J. P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M. J. Gait, L. V. Chernomordik and B. Leblue, "Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake," *J Biol Chem,* Vol. 278, No. 1, pp. 585-590, (2003).
67. I. W. Bauer, S. P. Li, Y. C. Han, L. Yuan, and M. Z. Yin, "Internalization of hydroxyapatite nanoparticles in liver cancer cells," *J Mater Sci Mater Med,* Vol. 19, No. 3, pp. 1091-1095, (2008).
68. S. Maeno, Y. Niki, H. Matsumoto, H. Morioka, T. Yatabe, A. Funayama, Y. Toyama, T. Taguchi and J. Tanaka, "The effect of calcium ion concentration on osteoblast viability proliferation and differentiation in monolayer and 3D culture," *Biomater,* vol. 26, no. 23, pp. 4847-4855, (2005).
69. M. Nagano, T. Nakamura, T. Kokubo, M. Tanahashi, and M. Ogawa, "Differences of bone bonding ability and degradation behaviour in vivo between amorphous calcium phosphate and highly crystalline hydroxyapatite coating," *Biomater,* Vol. 17, No. 18, 1771-1777, (1996).
70. P. Frayssinet, F. Tourenne, N. Rouquet, P. Conte, C. Delga, and G. Bonel, "Comparative biological properties of HA plasma-sprayed coatings having different crystallinities," *J Mater Sci Mater Med,* Vol. 5, No. 1, pp. 11-17, (1994).
71. G. Balasundaram, M. Sato, and T. J. Webster, "Using hydroxyapatite nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD," *Biomater,* Vol. 27, No. 14, 2798-2805, (2006).

72. Y. Zhao, Y. Zhang, F. Ning, D. Guo, and Z. Xu, "Synthesis and cellular biocompatibility of two kinds of HAP with different nanocrystal morphology," *J Biomed Mater Res B Appl Biomater*, Vol. 83, No. 1, pp. 121-126, (2007).

73. X. Feng, J. Ye, Y. Wang, and P. Rao, "Deagglomeration of HA during the precipitation synthesis," *J Mater Sci*, Vol. 40, No. 20, pp- 5439-5442, (2005).

74. M. Motskin, D. M. Wright, K. Muller, N. Kyle, T. G. Gard, A. E. Porter, and J. N. Skepper, "Hydroxyapatite nano and microparticles: correlation of particle properties with cytotoxicity and biostability," *Biomater,* Vol. 30, No. 19, pp. 3307-3317, (2009).